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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 14 September 1999 with an application for Letters Patent number 337792 made by DAVID WELLS.

Dated 27 September 2000.

Neville Harris
Commissioner of Patents

337792

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

NUCLEAR TRANSFER

I, DAVID WELLS a New Zealand citizen of 52 Enderley Avenue, Hamilton, New Zealand, do hereby declare this invention to be described in the following statement:

The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.

BACKGROUND

The stage of cell cycle of the donor nucleus and the recipient cytoplast at the time of embryo reconstruction are important factors determining successful development following nuclear transfer. Specific combinations between the two "cells" are required to ensure a diploid set of chromatin following the first embryonic cell cycle and to maximise the opportunities for nuclear reprogramming. When an interphase donor nucleus is fused with an enucleated metaphase-arrested oocyte, there is immediate nuclear envelope breakdown (NEBD) and the donor chromatin undergoes premature chromosome condensation (PCC)(Barnes *et al.*, 1993). These effects are induced by a cytoplasmic activity termed maturation promoting factor (MPF, alternatively called meiosis- or mitosis-promoting factor; see review by Campbell *et al.*, 1996a). MPF activity during oocyte maturation is maximal at metaphase stages and declines rapidly upon either fertilisation or artificial activation. Thus, two types of cytoplast may be used for reconstruction; those either high or low in MPF using either non-activated or activated Metaphase II (MII) cytoplasts, respectively.

The stage of the cell cycle of the donor nucleus and the length of exposure to MPF have marked effects on the degree of PCC observed. The chromatin of S-phase nuclei exposed to MPF has a typically pulverised appearance and chromosomal studies reveal a high incidence of abnormalities (Collas *et al.*, 1992b). In contrast, with nuclei at G1 or G2 the chromatin condenses to form elongated chromosomes with either single- or double-stranded chromatids, respectively (Collas *et al.*, 1992b). Following a suitable stimulus to release the reconstructed embryo from metaphase-arrest and to activate development, the nuclear envelope reforms around the donor chromatin which then

undergoes DNA synthesis regardless of its previous cell cycle stage. Thus, donor nuclei in G1 initiate DNA synthesis which is compatible with normal development, while nuclei in G2 or S-phases either completely or partially re-replicate already replicated DNA so that by the end of the first embryonic cell cycle the DNA content in the two daughter cells will be incorrect leading to abnormal early embryonic development.

In contrast, when nuclei are transferred to cytoplasts after the disappearance of MPF, following a sufficient interval after cytoplast activation, NEBD does not occur (so therefore neither does PCC) and it is the donor nucleus which controls DNA replication in accordance with its stage in the cell cycle at the time of transfer. Thus, nuclei in G1 or S-phases initiate or continue replication, respectively, while those in G2 are not induced to enter another round of DNA synthesis. Such pre-activated cytoplasts have been termed "universal recipients" (Campbell *et al.*, 1994) and are capable of co-ordinating the development of donor cells at any stage of the cell cycle. This has been especially important for cloning pre-implantation embryos where most undifferentiated nuclei are in S-phase (80-90%; Barnes *et al.*, 1993; Campbell *et al.*, 1994) and are therefore only compatible with transfer to cytoplasts low in MPF.

From the above discussion it is clear that in farm animals, at least, cytoplasts high in MPF are only compatible with diploid (2C) donor nuclei (*ie* those in either G0 or G1). However, two exceptions do exist in the mouse, at least. For example, embryonic blastomeres synchronised in metaphase (4C) are compatible with metaphase-arrested cytoplasts with a modification incorporating the use of cytoskeletal inhibitors to suppress polar extrusion body to generate two diploid "pronuclei" for serial transplantation which has ultimately resulted in live offspring (Kwon and Kono, 1996). In addition, mouse embryonic nuclei at the presumptive G2 stage (also 4C) are capable of generating diploid blastocysts following nuclear transfer, albeit at a low proportion, due to the expulsion of a diploid polar body removing the extra set of chromatin from the G2 donor nucleus (Cheong *et al.*, 1993).

Following nuclear transfer, normal development will depend upon factors present within the oocyte cytoplasm (or additional factors introduced exogenously) being able to remodel chromatin structure and to appropriately reprogramme the pattern of gene expression of the donor nucleus. The mature cytoplast contains the RNA transcripts and proteins to direct development of the normally fertilised cleavage-stage embryo up to the normal time of genome activation, when embryonic nuclei begin the synthesis of their own RNA to direct embryogenesis. Donor nuclei obtained from embryos or cell types which have already passed this point must therefore cease their RNA synthesis after reconstruction and remain inactive until the newly reprogrammed maternal-embryonic genome transition occurs. Following transfer, the donor nucleus is forced to reprogram to the zygotic state, and subsequently activate the appropriate genes at the correct levels, in the proper temporal and spatial manner for normal embryo development to occur. The mechanisms that achieve such nuclear reprogramming are not well understood.

The capacity of the donor chromatin to be remodelled and for gene expression to be reprogrammed, may depend upon the activity of cytoplasmic factors operating at the time of fusion. One factor of importance is the activity of MPF. With quiescent donor cells, however, the cytoplast stage appears to be less critical as comparable rates of embryo and fetal development have resulted from the use of cytoplasts with either high or low levels of MPF in both sheep (Campbell *et al.*, 1996b) and goats (Baguisi *et al.*, 1999). However, exposure to MPF may be more important for facilitating the reprogramming of differentiated cells which are in G1 as opposed to G0, as the chromatin in quiescent cells has already undergone some modifications and gene expression is greatly reduced.

The exposure of donor chromatin from somatic cells to cytoplasts high in MPF does not appear to be an absolute requirement for development, however. Vignon and colleagues (1998) reported that both quiescent (serum deprived) and actively cycling bovine skin and muscle cells fused to pre-activated cytoplasts (able to accept donor nuclei at any stage of the cell cycle) yielded blastocysts at a similar, albeit low, rate (2-5%) and generated both pregnancies and some live calves (Vignon *et al.*, 1999).

The first reports describing the production of cloned mammals from differentiated cells suggested the importance of using cells which had exited the normal cell division cycle and were synchronised in a quiescent or G0 state, based on absence of proliferating cell nuclear antigen (PCNA) indicating no cells in S-phase (Campbell *et al.*, 1996b; Schnieke *et al.*, 1997; Wilmut *et al.*, 1997; Patent WO 97/07669). Recently, however, it has been questioned as to what proportion of cells used in these studies where actually in G0 (based on data in the pig reported by Boquest *et al.*, 1999; see below).

Early studies with actively growing, unsynchronised cultures of embryonic cells (cell cycle stage unknown) fused to pre-activated cytoplasts did in fact produce lambs at term from early (Campbell *et al.*, 1995) but not later passage cells (Campbell *et al.*, 1996b). Subsequent studies, where the cells were deprived of serum for 5 days and reported to be quiescent (ie G0), yielded viable lambs following fusion with cytoplasts either before, after or simultaneous with activation at similar overall efficiencies (Campbell *et al.*, 1996b). Cibelli and colleagues (1998; and patent WO 99/01163) reported the use of actively growing cells fused to cytoplasts high in MPF in cattle, using randomly growing cells fused to MII cytoplasts which were subsequently activated using ionomycin and 6-dimethylaminopurine (6-DMAP). Importantly, however, it has not been proven that the donor cells which ultimately yielded the cloned calves originated from G1 cells. In theory, only cells in G0 or G1 are compatible with normal development with cytoplasts high in MPF in cattle. Cibelli *et al* (1998), reported that 82% of cells were positive for PCNA which is in contradiction to their flow cytometry results suggesting 52% were in G0/G1 (as they did not utilise markers to distinguish between G0 and G1 it is assumed that they refer to the proportion of diploid cells present). This discrepancy may lie with the PCNA staining method used (described by these authors in a concurrent publication; Zawada *et al.*, 1998) whereby cells were fixed in 2% paraformaldehyde. This fixative enables immunostaining of both soluble and insoluble fractions of PCNA which are present (albeit at low levels) at all stages of the cell cycle and also in quiescent cells, rather than detection only during S-phase (Bravo and MacDonald-Bravo, 1987).

The above discussion highlights the point that to date there has been generally poor characterisation of the stage of the cell cycle nuclei are in at the time of nuclear transfer which makes the state of the art uncertain and not easily replicated. Similarly, there are other reports whereby cloned calves (Vignon *et al.*, 1998, 1999; Zakhartchenko *et al.*, 1999) and mice (Wakayama and Yangimachi, 1999) have been produced from non-serum deprived cell cultures (where the vast majority of cells are assumed to not be in G0), however, the stage of the donor cell cycle at the time of nuclear transfer which resulted in the cloned offspring remains uncertain. Certainly none of these aforementioned studies (nor the patent filed by the University of Massachusetts WO 99/01163 on the use of non-quiescent cells) have demonstrated exactly what stage or stages of the cell cycle have resulted in that low proportion of reconstructed embryos which ultimately develop into viable offspring. One recent report (Boquest *et al.*, 1999) has been especially revealing, since the authors used dual parameter flow cytometry to simultaneously measure both cellular DNA and protein content (to distinguish between G0 and G1 cells in the diploid population, with quiescent cells having less RNA and protein) in order to investigate the cell cycle characteristics of cultured fetal pig fibroblasts. The results showed that, despite serum starvation for 5 days, less than 50% of cells where actually in G0 with another 40% synchronised in G1. In actively growing cultures on the other hand, 70% of all cells at any one time were in G1-phase (as this is typically the longest phase of the cell cycle with cultured somatic cells) and less than 3% were in G0. By selecting "small" cells in the population, the proportion in G0 increased to 72% and 7% for serum starved and cycling cells, respectively (Boquest *et al.*, 1999). This work highlights the importance of first establishing the cell cycle characteristics of different cultured cell lines (and verification after various cell cycle synchronisation treatments) to optimise cell cycle co-ordination for efficient nuclear transfer. It could be expected that different cell lines will have differing cell cycle dynamics, and it questions just what the proportions of G0 and G1 cells were in other studies using serum deprivation (*eg* Campbell *et al.*, 1996b; Wilmut *et al.*, 1997; Wells *et al.*, 1997; 1999ab).

It would therefore be desirable to have a method of nuclear transfer which ensured that the stage of cell cycle of the donor nuclei were accurately known.

It is an object of the present invention to go some way towards achieving this desideratum or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides an improved method of nuclear transfer, the improvement comprising selecting and segregating G1 cells from an actively growing population of donor cells at various unknown stages of the cell cycle and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell.

Such a method provides for the first time, certainty as to the state of the cell cycle of the donor nuclei and is therefore advantageous over the prior art.

According to a second aspect, the invention provides a method of nuclear transfer which comprises transferring a nucleus from a segregated G1 cell, into an enucleated recipient cell, whereby said G1 cell was segregated from a cell culture synchronised at an early G1 phase.

The segregated G1 donor cell is preferably from a cell culture. Suitable cells may be derived from either embryos, fetuses, juvenile animals, through to fully mature adults. Practically any diploid karyotypically normal cell that is capable of active cell proliferation could be used in the current invention. Cells could be of an undifferentiated cellular state or at any varying degree of cellular differentiation so long as they can be stimulated to enter the cell cycle and proliferate. Somatic cells which are naturally quiescent *in vivo* could be collected from donor animals and stimulated to enter the cell cycle with the appropriate culture conditions (such as by the addition of serum or specific growth factors) and used for nuclear transfer in an early G1 state following mitosis. Some cell types may well prove to be more efficient than others, however. It is expected that both adult and fetal fibroblasts will be satisfactory. By way of demonstration of the invention, results are presented below using granulosa cells collected from ovarian follicles from both a heifer and adult cow.

The methods of the present invention do not necessitate the use of potentially cyto-toxic or perturbing cell synchronisation agents such as nocodazole or colchicine to, for example, pre-synchronise cells in M-phase before subsequent release from this block and selection of cells in early G1 following cell division. However, in order to select larger numbers of cells from small cell populations it may be advantageous to utilise suitable reagents at appropriate drug concentrations. Furthermore, in the present invention, cells are preferably used in early G1 as opposed to late G1. So there is neither the requirement nor the desire to either wait a pre-determined length of time post-completion of mitosis or to use reagents such as aphidicolin to prevent entry into S-phase in order to use cells at the G1/S boundary.

According to a third aspect, the present invention provides a method of producing cloned mammalian embryos by transferring a segregated donor nucleus in early G1 phase into an enucleated recipient cell.

The methods of the present invention may be used to produce any animal species of commercial interest including birds, amphibia, fish and mammals. Preferably the animal of interest is a mammal, including, but not limited to, non-human primates, rodents, rabbits, cats, dogs, horses and most preferably, ungulates such as cattle, sheep, goats and pigs.

The methods of the present invention may be used to produce mammals having desirable genetic traits using genetically altered donor nuclei by methods well known in the art.

According to a fourth aspect, the present invention provides a reconstituted mammalian embryo prepared by the methods of the invention. The embryo so formed may then be serially re-cloned to further increase embryo numbers or aid reprogramming.

According to a fifth aspect, the present invention provides a method of cloning a mammal comprising (1) producing a cloned mammalian embryo according to the method of the invention described above; (2) allowing a mammal to develop to term from the embryo

by known methods; and (3) optionally breeding from the mammal so formed either by conventional methods or by cloning according to the methods of the present invention.

According to a sixth aspect, the present invention provides a cloned mammal prepared by the methods of the invention, described above.

The present invention will now be described by reference to the following examples in the detailed description which are provided for the purposes of illustration only and do not limit the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an improvement of the known techniques for the cloning of mammalian embryos by nuclear transfer. Although it is contemplated that the embryo cloning procedure of the present invention may be utilised in a variety of mammals and, indeed other animal species, the procedure will be described with reference to the bovine species. It is an essential feature of this invention that the donor nuclei are in G1 phase and, preferably, in early G1 phase.

One approach in obtaining G1 cells with certainty is to individually pick mitotic cells from the culture surface, allow them to complete mitosis in medium containing 10% FCS and to fuse the individual cells to cytoplasts within a short period of time following mitosis, such as three hours in common practice. In this way they are assured of being in the early phase of G1. Thus, the cycling cells are used for nuclear transfer well before they have progressed to the G1/S boundary. The selected cells remain in high serum-containing medium throughout the manipulations, at least until after fusion with the cytoplast has been completed. The only exceptions are when the cells are washed in phosphate-buffered saline (PBS) and exposed temporarily to a weak enzymatic solution to facilitate their removal from the culture surface without excessive physical damage prior to cell injection. Thus, the cells are not induced to exit the cell cycle and do not become quiescent at any point.

For improved development of the cloned embryos it is preferable to expose the donor nucleus (introduced either following electrically-induced cell fusion or direct nuclei injection) to factors present within the cytoplasm of the enucleated oocytes for a suitable period of time in order to facilitate nuclear programming. This has been termed "fusion before activation" or FBA. Previous work has demonstrated the benefits of this approach compared to essentially "simultaneous fusion and activation" or AFS (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999b). Furthermore, it is recommended that exposure to the cytoplasm be at least greater than one hour duration (see results) and commonly between 3-6 hours in order to improve rates of development to the blastocyst-stage. With this method, however, it is important to prevent by some suitable means the micro-nuclei formation which occurs when fusion precedes activation (Czolowska *et al.*, 1984) in order to maintain the correct ploidy in the resulting embryo.

Below is outlined a method for reconstructing and producing cloned embryos, in the bovine species, derived from both G0 (control) and G1 cultured donor cells. In this particular example, bovine granulosa cells collected from ovarian follicles were used. In practice, essentially any cell type possessing a normal diploid karyotype, including embryonic, fetal, juvenile and adult cells, which is either actively proliferating or can be induced to enter the cell cycle may prove totipotent using this technology. In addition, any other method known in the art may be used to reconstitute and produce cloned embryo's as would be appreciated by a person skilled in the art.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from mature cows and placed in saline (30 °C) and transported within 2 hours to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3-10 mm follicles using an 18-gauge needle and negative pressure. (Alternatively, immature oocytes could be collected from donor cows via ovum pick-up and subsequently matured *in vitro*). COCs were collected into HEPES-buffered Tissue Culture Medium 199 (H199; Life Technologies, Auckland, New Zealand) supplemented with 50 μg/ml heparin (Sigma, St. Louis, MO) and 0.4% w/v BSA

(Immuno-Chemical Products (ICP), Auckland, New Zealand). Before *in vitro* maturation, only those COCs with a compact, non-atretic cumulus oophorous-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered Tissue Culture Medium 199 medium + 10% FCS. Ten COCs were transferred in 10 µl of this medium and placed into a 40 µl drop of maturation medium in 5-cm petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised Tissue Culture Medium 199 supplemented with 10% FCS, 10 µg/ml ovine FSH (Ovagen; ICP), 1 µg/ml ovine LH (ICP), 1 µg/ml oestradiol (Sigma), and 0.1 mM cystamine (Sigma)(de Matos *et al.*, 1995). Microdrop dishes were cultured at 39 °C in a humidified 5% CO_2 in air atmosphere for 18-20 hours. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered Synthetic Oviduct Fluid (HSOF; Thompson *et al.*, 1990) for 3 minutes, followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Cultured Cells

a) **Media** Matured oocytes, cytoplasts and reconstructed embryos were either held or manipulated in H199-based media for the period following maturation and until fusion was assessed 15-30 minutes after the electrical pulses. Embryos that were reconstructed by fusing donor cells and MII cytoplasts 3-6 hours before activation (FBA treatment) were cultured in Synthetic Oviduct Fluid (SOF; Gardner *et al.*, 1994) minus calcium + 10% FCS until just before activation some 3-6 hours later. Following this point, calcium was present in all media formulations used as was the case for those embryos activated within one hour of fusion of the donor cell (see "Activation" below).

b) **Enucleation** Oocytes matured for approximately 18-20 hours were enucleated with a 20-30 µm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were previously stained in H199 medium containing 10% FCS, 5 µg/ml Hoechst 33342 and 7.5 µg/ml

cytochalasin B (Si[redacted]) for 20 minutes and manipulate[redacted] this medium but without Hoechst 33342. Enucleation was confirmed by visualising the karyoplast under ultraviolet light. Following enucleation, the resulting cytoplasts were washed extensively in H199 + 10% FCS and held in this medium until injection of donor cells.

c) Preparation of quiescent (G0) donor cells Cultured granulosa cells were induced to enter a period of quiescence (presumptive G0) by serum deprivation (Campbell *et al.*, 1996b). One day after routine passage, the culture medium was aspirated and the cells washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The granulosa cells were returned to culture for a further 9-23 days (commonly 10 days) in low serum before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in H199 + 0.5% FCS and remained in this medium until injection.

d) Preparation of G1 donor cells Actively growing granulosa cells were cultured in an appropriate medium (for example DMEM/F12 plus 10% fetal calf serum) on glass coverslips in the culture dish. These coverslips containing the cells growing on their surface may be physically picked out of the culture dish in a sterile manner and placed into a suitably constructed micro-manipulation chamber, to enable cell or nuclei collection and injection. A droplet of HEPES-buffered medium containing 10% FCS is initially placed onto the cells which is then overlaid with mineral oil. When one is ready to begin harvesting cells, the medium is aspirated and the cell layer washed with PBS before introducing a dilute concentration of trypsin solution (such as at one-tenth the strength used for routine sub-cultivation of the cultured cell line) containing 1.5 µg/ml cytochalasin B, principally to minimise mechanical damage from the physical removal of the cells from the coverslip. With suitable microscopy (for example phase contrast optics) mitotic cells are identified on the coverslip primarily by visualising condensed chromatin on a mitotic spindle. Thus, there is no need to use a DNA specific flurochrome such as Hoescht 33342 and expose cells to UV light. With the aid of the injection pipette mounted on the manipulator, these mitotic cells are individually picked off the coverslip and placed into

an adjacent droplet of medium containing 10% FCS to enable complete mitosis and eventual cell cleavage to form a doublet of cells. Thus, cells in metaphase, or preferably anaphase or telophase are individually selected, removed and allowed to complete mitosis and cleave in two. These cell doublets are then gently separated into individual cells which may be easily achieved by brief exposure to a suitable enzymatic solution. Each intact cell is then injected and fused to the cytoplast. Alternatively the cell nucleus may be isolated and injected directly into the cytoplasm of the enucleated oocyte. Preferably this is completed within three hours of originally picking the mitotic cell off the culture surface. This ensures that the cultured cells are fused at an early G1 stage of the cell cycle and well before S-phase occurs.

e) **Microinjection** Recipient cytoplasts were dehydrated in H199 containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A 30-35 μm pipette (external diameter), containing the donor cell, was introduced through the same slit in the zona pellucida as made during enucleation and the cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. Following injection, the reconstructed embryos were rehydrated in two steps; firstly in H199 containing 10% FCS and 2.5% sucrose for 5 minutes and then in H199 + 10% FCS until fusion.

f) **Cell fusion** Reconstructed embryos in the FBA group were electrically fused at approximately 24 hours post-start of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES and 0.05% fatty acid free (FAF) BSA with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 μm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 2.25 kV/cm for 15 μs each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). Following the electrical stimulus, the reconstructed embryos were washed in H199 + 10% FCS. They were then checked for fusion by microscopic examination within 15-30 minutes.

The electrical fusion parameters above are not expected to cause significant rates of activation with young cytoplasts used at 24 hpm, since less than 1% of control oocytes (n=112) at the same age formed pronuclei after a similar electrical stimulus (Wells *et al.*, 1999b). This is important for the FBA treatment, so that NEBD and PCC occur, allowing exposure of the donor chromatin of the G1 and G0 nuclei to reprogramming factors present within the oocyte cytoplasm.

Alternatively, the nuclei from cells in G1 may be isolated and injected directly into the oocyte cytoplasm as known by a person skilled in the art.

g) Activation There are a variety of methods to effect artificial activation. One particular method involves the combination of ionomycin (Sigma) and 6-dimethylaminopurine (6-DMAP; Sigma) (Susko-Parrish *et al.*, 1994). Following fusion, embryos were either activated immediately (or at least within 1 hour of fusion) or preferably, donor nuclei were exposed to the oocyte cytoplasm for a period of 3-6 hours following fusion before chemical activation. This preferred method has been termed "fusion before activation" (FBA; Wells *et al.*, 1998). Thirty minutes before activation, fused embryos in the FBA treatment were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30 µl drops of 5 µM ionomycin (Sigma) in HSOF + 1mg/ml FAF BSA for 4 minutes at 37 °C. Activation commonly occurred in cytoplasts aged between 27-30 hpm. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 minutes before culture in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) for 4 hours in SOF (plus calcium) + 10% FCS.

The improved rates of embryo development that result from a period of prolonged exposure of the nucleus to oocyte cytoplasm as in the FBA methodology (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999b) must be combined with suitable treatments to prevent micronuclei formation occurring following such delayed activation (Czolowska *et al.*, 1984). A serine-threonine kinase inhibitor such as 6-DMAP appears to be one suitable reagent possibly inhibiting phosphorylations necessary for the spindle apparatus. 6-DMAP

therefore allows for the formation of a single intact nucleus following the initial activation stimulus thus, maintaining the correct ploidy in the reconstructed embryo.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20 µl drops of SOFaaBSA (8 mg/ml FAF BSA) (Gardner *et al.*, 1994) overlaid with paraffin oil. Whenever possible, groups of up to 10 embryos were cultured together. Embryos were cultured in a humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39 °C in a 5% CO_2: 7% O_2: 88% N_2 gas mix. On Day 5, embryos were transferred to fresh 20 µl drops of SOFaaBSA + 10% charcoal-stripped FCS (csFCS) (Thompson *et al.*, 1998). On Day 7 post fusion, development to transferable-quality blastocysts assessed.

An alternative culture system described in New Zealand specification no. 333595 may be used to further increase development to transferable blastocysts.

RESULTS

Table 1 The effect of cell cycle stage (either presumptive G0 or definitive G1) and exposure of donor nuclei to oocyte cytoplasm before activation on development following nuclear transfer. Data are presented on transferable quality blastocysts produced after seven days of *in vitro* culture and the post-transfer viability of these cloned embryos on Days 23-26, 60 and 100 of gestation and at term, using granulosa cell lines from a heifer (J1) and adult cows (LFC and EFC).

		Blastocyst development		Viability following embryo transfer			
cell line	cell stage	Act<1hpf[1]	Act 3-6hpf[2]	Day 23-26[3]	Day 60[4]	Day 100[4]	viable calves
LFC[5]	G0	6/85 (7)	106/412 (26)	ND	22/74 (30)	17/74 (23)	6/74 (8)
EFC[6]	G0	ND	152/552 (28)	23/29 (79)	45/100 (45)	21/100 (21)	10/100 (10)
EFC	G1	4/41 (10)	30/90 (33)	8/13 (62)	ND	ND	ND
J1	G0	ND	17/68 (25)	2/7 (29)	ND	ND	ND
J1	G1	ND	129/334 (39)	1/9 (11)	8/66 (12)	7/66 (11)	ND

1 reconstructed embryos activated within one hour post fusion (hpf)

2 reconstructed embryos activated between three and six hours post fusion (hpf)

3 concepti recovered following slaughter of recipients receiving a "bulk embryo transfer"

4 fetuses detected by ultrasonography at approximately these gestational ages

5 & 6 these data provided for comparison and are obtained from experiments described in Wells *et al.*, 1999a and b, respectively.

ND not determined

CONCLUSIONS

1. It is possible to select individual cells at a definitive stage of the cell cycle, namely early G1. This is advantageous compared to prior art using so-called actively proliferating cells where the actual stages of the cell cycle are not accurately known for each individual cell used for nuclear transfer. Furthermore, the preferential use of early G1 cells may result in improved efficiency compared to cells at late G1 stages.
2. Post-mitotic cells in the early G1 phase of the cell cycle are totipotent following nuclear transfer.

3. Thus, G0 is not the only stage of the cell cycle that is compatible with development following nuclear transfer with differentiated cultured cells and early G1 nuclei can also be functionally reprogrammed.
4. Post-mitotic early G1 cells promote development to the blastocyst stage following nuclear transfer to similar levels as cells in presumptive G0.
5. Initial data suggests that the viability post transfer may be lower than with G0 cells

It will be appreciated that the description is not intended to limit the scope of the invention to the above examples only, many variations such as might readily occur to a person skilled in the art being possible, without departing from the scope thereof.

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